Article

A new phytotelm-breeding treefrog of the genus *Nasutixalus* (Rhacophoridae) from western Yunnan of China

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Abstract

A new treefrog of the genus *Nasutixalus* is described from montane evergreen forest in Yingjiang County, in western Yunnan, China. The new species, *Nasutixalus yingjiangensis* **sp. nov.**, can be distinguished from its congeners by a combination of the following characters: medium body size (SVL 39.5–40.0 mm in adult males, 47.5 mm in a single female); tympanum indistinct and covered with tubercles; disc diameter of third finger greater than tympanum diameter; dorsal skin relatively smooth, scattered with small tubercles, those on head and anterior dorsum of body more dense and more prominent; light brown above with a dark brown marking between eyes and two broad dark brown lateral strips on the dorsum; iris with a weak "X"-shaped, light colored marking; interorbital distance shorter than the upper eyelid width; comparatively short foot (mean TFL/SVL ratio 67.0% and 62.9% in males and female respectively). The new species appears to be forest-dependent and was found in well-preserved montane rainforest; it inhabits the canopy and breeds in tree holes during the rainy season. The type locality of the new species is an isolated forest fragment surrounded by degraded landscape and efforts are already underway to protect the site. *Nasutixalus yingjiangensis* **sp. nov.** represents the third known species of the newly established genus *Nasutixalus*.

Key words: new species, taxonomy, molecular, *Nasutixalus yingjiangensis* **sp. nov.**, Yingjiang County

Introduction

The old world treefrogs of the family Rhacophoridae are currently one of the largest families of amphibians, containing over 400 recognized species (Frost 2017). To date, 18 genera are recognized within the family, with seven of them only recognized in the past decade (Frost 2017). The newly described rhacophorid genus *Nasutixalus* was established by Jiang *et al*. (2016) on the basis of evidence from morphological analysis and the phylogenetic position of its type species, *N. medogensis* Jiang, Wang, Yan & Che from Medog (=Motuo) in southeast Tibet. Phylogenetically, the genus *Nasutixalus* was reconstructed as a member of the clade containing *Kurixalus*, *Philautus*, *Beddomixalus*, *Mercurana*, *Pseudophilautus* and *Rarochestes* (Jiang *et al.* 2016). Almost concurrently, Biju *et al*. (2016) also described a new genus of Rhacophoridae, *Frankixalus*, based on phylogenetic, morphological, osteological and ecological studies, and assigned *F. jerdonii* (Günther) from north-eastern India as the type species of the new genus. The phylogenetic data presented in Biju *et al.* (2016) indicated that *Frankixalus* was sister to a clade encompassing *Kurixalus*, *Beddomixalus*, *Mercurana*, *Raorchestes* and *Pseudophilautus*; their analysis also indicated the presence of an unnamed species, referred to as *Frankixalus* sp., from southern Tibet of China (= described as *N. medogensis* in Jiang *et al*. 2016). Apparently, these two new genera belong to the same phylogenetic lineage, and therefore *Frankixalus* is objectively invalid and regarded as a junior synonym of *Nasutixalus* based on publication priority (see Sivongxay *et al.* 2016). As a result, two species of the genus *Nasutixalus* are hitherto recognized and recorded from the eastern Himalayas, i.e., *N. jerdonii* from northeast India, and *N. medogensis* from southeast Tibet of China. Both species are reported to inhabit the canopy in montane evergreen forest at elevations above 1300 m a.s.l. (Biju *et al*. 2016; Jiang *et al*. 2016); *N. jerdonii* was reported to be a tree hole breeder with peculiar oophagous tadpoles (Biju *et al*. 2016).

During field surveys between July 2016 and June 2017, we collected specimens of an unknown treefrog from

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Yingjiang County, Yunnan Province, China. The treefrog inhabits the upper tree layer and breeds in tree hole, sharing the same niche and breeding habits as reported for *N. jerdonii* (Biju *et al*. 2016). Subsequent morphological and molecular studies further revealed that the species is assigned to the genus *Nasutixalus*. However, this species can be distinguished from the two known congeners and other rhacophorids by a combination of morphological characters and molecular divergence; we therefore describe it herein as a new species.

Materials and methods

Sampling. Specimens were collected during fieldwork in Yingjiang County, Yunnan Province of China, fixed and preserved in 80% ethanol, and deposited at the Museum of Biology, Sun Yat-sen University (SYS). The geographic position of the surveyed locality and the distribution records of the two other known *Nasutixalus* species are shown in Fig. 1.
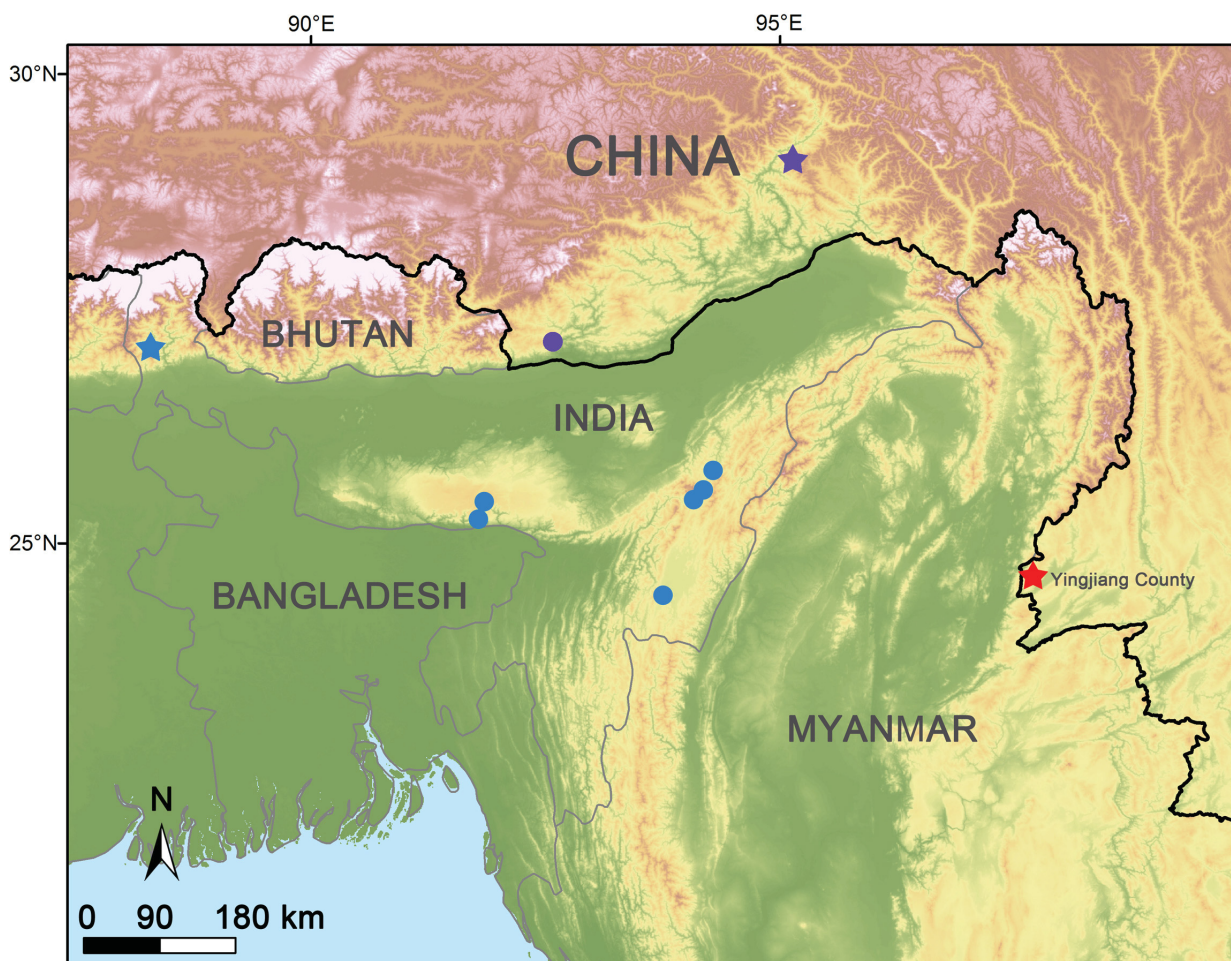
FIGURE 1. Map showing the known distribution records of the three recognized species of *Nasutixalus* (refers to Biju *et al.* 2016; Jiang *et al*. 2016). Red: *Nasutixalus yingjiangensis* **sp. nov.** in Yingjiang County of Yunnan Province, China; blue: *Nasutixalus jerdonii* in India; purple: *Nasutixalus medogensis* in Tibet of China. Stars indicate the type localities of each species.

DNA extraction and sequencing. Genomic DNA was extracted from the muscle tissue using TIANamp Genomic DNA Kit. We amplified fragments of the mitochondrial 12S rRNA and 16S rRNA from our *Nasutixalus* sp. specimens using the primer pairs L1091/H1478 and L3975/H4551 (Simon *et al.* 1994; Chen *et al*. 2013) respectively. PCR amplifications were performed in a reaction volume of 25 μl containing 100 ng of template DNA, 0.3 mM of each PCR primer and 10 μl Premix EX TaqTM (Takara). The PCR conditions were an initial denaturing step at 95 °C for 4 mins, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30

s an extension step at 72 °C for 1 min, and a final extension of 72 °C for 7 mins. PCR products were purified with spin columns. The purified products were sequenced with both forward and reverse primers using BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, Mass., USA) according to the guidelines of the manufacturer. The products were sequenced on an ABI Prism 3730 automated DNA sequencer at the ShangHai Majorbio Bio-pharm Technology Co.,Ltd.

TABLE 1. Samples and sequences used in the phylogenetic analysis in this study.

Species	Locality	Voucher No.	GenBank No. (12S, 16S)
(1) *Nasutixalus* sp.	Yingjiang, Yunnan, China	SYS a005802	MG603585, MG603588
(2) *Nasutixalus* sp.	Yingjiang, Yunnan, China	SYS a005803	MG603586, MG603589
(3) *Nasutixalus* sp.	Yingjiang, Yunnan, China	SYS a005804	MG603587, MG603590
(4) *Beddomixalus bijui*	Kadalar, Kerala, India	SDBDU 2011.1006	KU170017, KU169995
(5) *Buergeria oxycephala*	Hainan, China	SCUM 050267YJ	EU215524
(6) *Chiromantis nongkhorensis*	Laos	FMNH 255378	Q204774, GQ204723
(7) *Feihyla palpebralis*	Lam Dong, Vietnam	KIZ 712	GQ285681
(8) *Ghatixalus variablilis*	Mukkurthi NP, Tamil Nadu, India	SDBDU 2008.4409	KU170006, KU169981
(9) *Gracixalus gracilipes*	Mt. Dawei, Yunnan, China	060821196RAO	GQ285668
(10) *Kurixalus odontotarsus*	Mengyang, Jinghong, China	SCUM 060688L	EU215549
(11) *Liuixalus ocellatus*	Mt. Wuzhi, Hainan, China	HN0806045	GQ285672
(12) *Mercurana myristicapalustris*	Chathankod, Kerala, India	SDBDU 2011.849	KU170016, KU169994
(13) *Nasutixalus jerdonii*	Raenghzaeng, Manipur, India	SDBDU 2009.46	KU170018, KU169996
(14) *Nasutixalus jerdonii*	Cherrapunjee, Meghalaya, India	SDBDU 2009.271	KU230453, KU169997
(15) *Nasutixalus jerdonii*	Mawphlang, Meghalaya, India	BNHS 5977	KU230454, KU169998
(16) *Nasutixalus jerdonii*	Mawphlang, Meghalaya, India	SDBDU 2009.1166	KU230455, KU169999
(17) *Nasutixalus jerdonii*	Tseminyu, Nagaland, India	SDBDU 2009.362	KU230456, KU170000
(18) *Nasutixalus jerdonii*	Sechüma, Nagaland, India	SDBDU 2007.054	KU230457, KU170001
(19) *Nasutixalus jerdonii*	Sechüma, Nagaland, India	SDBDU 2007.055	KU230458, KU170002
(20) *Nasutixalus jerdonii*	Meriema, Nagaland, India	SDBDU 2007.060	KU230459, KU170003
(21) *Nasutixalus medogensis*	Sessa, Arunachal Pradesh, India	SDBDU 2009.1295	KU230460, KU170004
(22) *Nasutixalus medogensis*	Sessa, Arunachal Pradesh, India	SDBDU 2009.1261	KU230461, KU170005
(23) *Nasutixalus medogensis*	Motuo, Xizang, China	6255Rao	GQ285679
(24) *Nasutixalus medogensis*	Motuo, Xizang, China	KIZ016395	KU243082
(25) *Nyctixalus pictus*	Sarawak, Malaysia, Borneo	MVZ 239460	GQ204783, GQ204732
(26) *Philautus aurifasciatus*	Java, Indonesia		AY141805, GQ204702
(27) *Polypedates megacephalus*	Mt. Daiyun, Fujian, China	SCUM 050508C	EU215552
(28) *Pseudophilautus microtympanum*	Central Hills, Sri Lanka	WHT5065	AF249030, GQ204678
(29) *Raorchestes glandulosus*	Coorg, Karnataka, India	SDBDU 2011.817	KU170012, KU16998
(30) *Rhacophorus reinwardtii*	Java	ZRC 1.1.5273	GQ204771, GQ204720
(31) *Taruga fastigo*	Sri Lanka	WHT2783	Y141802, GQ204690
(32) *Theloderma asperum*	Malaysia	ZRC 1.1.9321	GQ204776, GQ204725
(33) *Limnonectes poilani*	Vietnam	AMNH A163717	DQ283378

Phylogenetic analyses. In addition to the newly collected specimens, sequences of other two species of the genus *Nasutixalus*, as well as representatives of each currently recognized genera of the family Rhacophoridae, were included in the genetic analysis; *Limnonectes poilani* was used as an outgroup after Biju *et al.* (2016) and Jiang *et al.* (2016) (Table 1). The resulting sequences were aligned by Muscle 3.6 (Edgar, 2004) under default

settings and then checked by eye and adjusted if necessary. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood analysis was conducted in RAxML v8.2.4 (Stamatakis 2014). Confidence intervals were determined with 1000 bootstrap replicates utilizing the rapid bootstrap option under the GTRGAMMA substitution model. Bayesian analyses were performed in MrBayes 3.2.6 (Ronquist *et al.* 2012) under the GTR+I+G model as suggested by the Akaike Information Criterion implemented in jModelTest 2.1.2 (Darriba *et al.* 2012). We employed two separate MCMC runs, each with four Metropolis-coupled chains. The analyses were run for 2.5 million generations, with parameters and topology sampling every 1000 generations. The stationary phase was detected using Tracer 1.6 (Rambaut *et al.* 2014). The first 1000 trees were discarded as burn-in and posterior probabilities were determined from the remaining trees. We also calculated the uncorrected pairwise genetic distances using MEGA 6.06 (Tamura *et al.* 2013).

Morphological characters. Measurements followed Fei *et al.* (2009) and Biju *et al.* (2016), and were taken with digital callipers to the nearest 0.1 mm: SVL = snout-vent length; HL = head length from tip of snout to the articulation of the jaw; HW = head width at the greatest cranial width; SL = snout length, from tip of snout to the anterior corner of the eye; IND = internasal distance; IOD = interorbital distance, closest distance between the upper eyelids; UEW = maximum upper eyelid width; ED = eye diameter, from the anterior corner of the eye to the posterior corner of the eye; TD = tympanum diameter; TED = tympanum-eye distance, from the anterior edge of tympanum to the posterior corner of the eye; LAHL = length of lower arm and hand; HAL = hand length, from the carpal-metacarpal articulations to the tip of the longest finger; HLL = hindlimb length; FML = femur (thigh) length; TIB = tibia (shank) length; LFT = length of foot and tarsus; FL = foot length; FDW = finger disc width; FW = finger width, measured at the base of the disc; TDW = toe disc width; TW = toe width, measured at the base of the disc; digit number is represented by roman numerals I–V. Webbing formulae are given following Savage (1975) and Biju *et al.* (2016). Sex was determined by the presence of nuptial pads. Comparative morphological data of two known species of *Nasutixalus* were obtained from the literature: Biju *et al.* (2016) and Jiang *et al.* (2016).

Acoustic analyses. The call of an unvouchered individual of the new species was recorded using a TASCAM DR-40 digital sound recorder (44.1 kHz sampling rate and 24-bit encoding), held approximately 3 m from the calling individual at an ambient temperature of 15 °C at 21:30 h on 21 April 2017. Calls were analysed with Raven Pro © v.1.5 software (The Cornell Lab of Ornithology, available from http://www.birds.cornell.edu/raven). The sonograms and waveforms were generated by Raven Pro 1.5 software with Fast Fourier samples 512 points and overlap 50%, from which all parameters and characters were measured. For each call recording, we measured the call duration (s), intercall-interval (s), call repetition rate (calls/min), and dominant frequency (kHz).

Results

Phylogenetic analyses. Two partial sequences of 12S and 16S rRNA genes were combined as a 1266 bp fragment for the phylogenetic analyses. Both the ML tree and BI analyses based on the fragment combined strongly support the placement of the unknown rhacophorid specimens from Yingjiang County in the genus *Nasutixalus* (Fig. 2) and substantially separate these samples from all currently recognized genera of Rhacophoridae by a large uncorrected sequence divergence (Table 2). The *Nasutixalus* sp. from Yingjiang County is reconstructed as a sister species of *N. jerdonii*, whereas *N. medogensis* is a sister lineage to them. For the uncorrected pairwise divergence in the 12S and 16S rRNA genes fragment, there are significantly high genetic distances between the *Nasutixalus* sp. from Yingjiang and the other two known congeners, *p* = 7.1–7.5% in between with *N. jerdonii*, and *p* = 7.9% in between with *N. medogensis*, while the intraspecific genetic distances between *N. jerdonii* and *N. medogensis* are 9.8–10.2%. These levels of divergences are typically indicative of differentiation at the species level in amphibians (Vences *et al.* 2005). As the observed molecular differences are corroborated by differences in morphology (see below), we therefore consider the *Nasutixalus* sp. from Yingjiang County to be an undescribed species.

Nasutixalus yingjiangensis sp. nov.

Holotype. SYS a005802, adult male, calling from inside a small tree hole on a small tree (*Tetradium glabrifolium*, ca. 13 cm in diameter at breast height) in a montane evergreen broadleaf forest at Tongbiguan Town, Yingjiang

County, Yunnan Province, China (24°37'21.59" N, 97°37'13.29" E, 1610 m above sea level), collected on 20 April 2017 by J.H. Yang.

Paratypes. SYS a005803, adult male; SYS a005804, adult female, both from the same locality as holotype, collected on 10 June 2017 by J.H. Yang, Shen-Pin Yang, Li-Yan Wang and Rong-Jia Li.

Etymology. The specific name "*yingjiangensis*", is a Latinized toponymic adjective in reference to the type locality of the new species, Yingjiang County of Yunnan Province, China. For the common names, we suggest "Yingjiang Tree-hole Frog" (English) and "Yíng jiāng léng bí shù wā" (Chinese).

Diagnosis. The new species is assigned to the genus *Nasutixalus* by the possession of the following morphological characters considered to be diagnostic for the genus: medium body size ; outline of snout truncate in dorsal view, semi-circular in ventral view, slightly vertical in lateral view; snout not protruding; canthus rostralis obtuse and raised prominently, forming a ridge from nostril to anterior corner of eyes; webbing rudimentary on fingers and well developed on toes; dorsal skin relatively smooth, scattered with small tubercles; iris with a weak "X"-shaped, light colored marking (Biju *et al.* 2016; Jiang *et al.* 2016), and on the basis of molecular analyses (see above and Fig. 2).
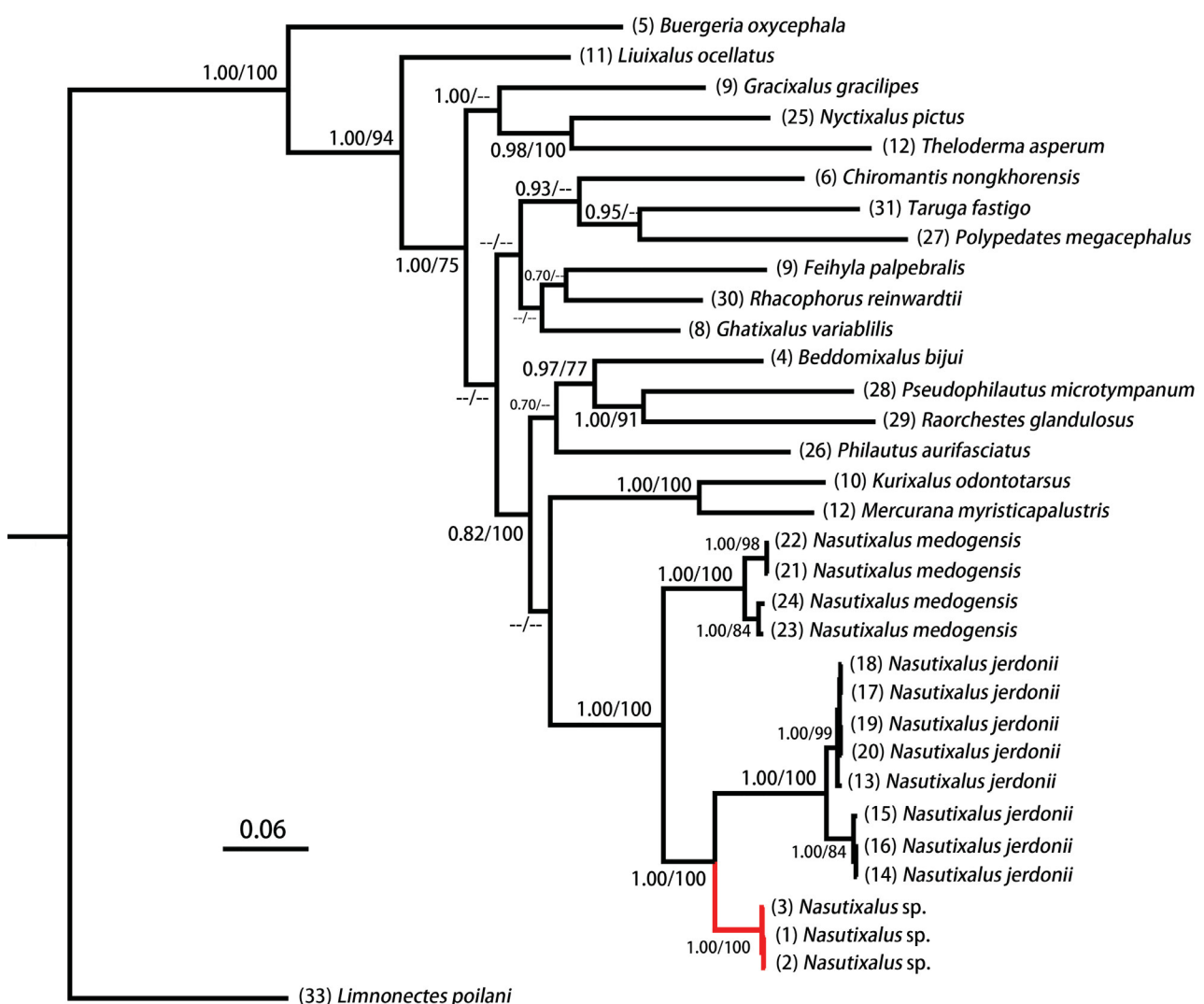
FIGURE 2. Bayesian inference (BI) tree derived from 1266 bp sequences of the mitochondrial 12S and 16S rRNA genes. Numbers above branches are Bayesian posterior probabilities (> 70% retained) and numbers below branches indicate bootstrap support values for maximum likelihood analyses (>70% retained).

Nasutixalus yingjiangensis **sp. nov.** can be distinguished from its congeners by a combination of following morphological characters: medium body size (SVL 39.5–40.0 mm in adult males, 47.5 mm in a single female);

tympanum indistinct and covered with tubercles; disc diameter of third finger greater than tympanum diameter; dorsal skin relatively smooth, scattered with small tubercles, those on head and anterior dorsum of body more dense and more prominent; light brown above with a dark brown marking between eyes and two broad dark brown lateral strips on the dorsum; iris with a weak "X"-shaped, light colored marking; interorbital distance shorter than the upper eyelid width; comparatively short foot (mean TFL/SVL ratio 67.0% and 62.9% in males and a single female respectively).

Description of holotype (measurements in mm). SYS a005802 (Fig. 3 & 4), adult male, medium body size (SVL 40.0), body habitus moderate; head width (HW 15.4) slightly greater that head length (HL 13.8); snout length (SL 5.8) nearly equal to horizontal diameter of the eye (ED 5.4); snout truncated in dorsal view, semi-circular in ventral view, and nearly slightly vertical in lateral view, not protruding; canthus rostralis rounded and raised noticeably, forming a ridge from nostril to anterior corner of eye; loreal region slightly concave; interorbital distance (IOD 3.7), slightly shorter than width of the upper eyelid (UEW 4.0), and internasal distance (IND 4.0); nostrils notably anterolaterally protuberant, oval, slightly closer to tip of snout (NS 2.4) than to eye (EN 2.6); tympanum barely distinct and circular (TD 2.2), 0.42 times of eye diameter, distance from eye (TED 0.7) by about one third of its own diameter; vomerine ridge distinct, obtuse, closer to choanae than each other; vomerine teeth absent; tongue deeply emarginated without median lingual process; supratympanic fold distinct and gently curved ventrally, extending from behind the eye, over the tympanum to axilla.

Forelimbs moderately long, robust, lower arm and hand length (LAHL 20.6) about half of body length; relative length of fingers I<II<IV<III; all finger tips dilated with well-developed disks with distinct circummarginal grooves (FDWI 1.6, FWI 1.3; FDWII 2.3, FWII 1.3; FDWIII 2.5, FWIII 1.3; FDWIV 2.2, FWIV 1.4), third finger disk width slightly larger than tympanum diameter; all fingers with distinct lateral fringes on the inner and outer sides, webbing on fingers rudimentary ($I2^{+}$–$2^{+}II2$–$3III2^{2}/_{3}$–$2^{1}/_{2}IV$); subarticular tubercles prominently domed, rounded, formula 1, 1, 2, 2; thenar tubercles well developed; inner and outer metacarpal tubercles distinct, oval and long, inner one greater than outer one; distinct small supernumerary tubercles present on the base of all fingers; distinct nuptial pad present, surface microgranular, covering the dorsal surface of the basal phalange of finger I.

Hindlimbs long and slender (HLL 61.0), 171% of SVL; tibiotarsal articulation reaching the posterior corner of eye when adpressed along the body; shank (TIB 18.4) slightly longer than thigh (FML 18.0), and slightly shorter than foot (FL 18.9, LFT 26.6); toes moderately long and thick, relative lengths I<II<III<V<IV; tips of toes with well-developed disks with distinct circummarginal grooves, disks slightly smaller than those of fingers; relative width of discs I<II=III<V<IV (TDWI 1.5, TWI 1.0, TDWII 2.0, TWII 1.5, TDWIII 2.0, TWIII 1.4, TDWIV 2.4, TWIV 1.5, TDWV 2.1, TWV 1.3); all toes with distinct lateral fringes on the inner and outer sides, toe webbing moderate ($I1^{1}/_{3}$–$2II1$–$2^{+}III1$–$2^{+}IV2$–$1V$); subarticular tubercles distinctly domed, and rounded formula 1, 1, 2, 3, 2; inner metatarsal tubercle prominent, oval and long (2.1 in length); outer metatarsal tubercle absent; small supernumerary tubercles presents on the base of all toes; tarsal glandular ridge absent.

Dorsal surfaces of head, body and limbs smooth and scattered with small tubercles, those on dorsal head, eyelids and anterior body relatively prominent and dense whilst those on posterior body, lower flanks and limbs relatively weak and scarce; lateral surfaces of head covered with small tubercles, those on temporal region and supratympanic fold relatively prominent and dense; tympanum finely covered with tubercles; ventral surfaces of throat, chest, belly and basal thighs densely covered with distinct flat tubercles, those on throat and chest relatively smaller; ventral surfaces of the forelimbs, shanks and tarsus scattered with small tubercles.

Coloration of holotype in life. Dorsal surface pale brown with a moderate-sized dark inverted triangular-shaped blotch between the eyes extending to the upper eyelids, and two relatively broad dark lateral stripes that extend from above scapular region to groin; the two dark stripes on dorsum not in contact and anteriorly separated from the dark interorbital blotch between eyes; dark brownish broad transverse bands present on dorsal surface of fore and hind limbs: three on the lower arm, three on the thigh and tibia; a dark line extends from snout, through nostril and along canthus rostralis to the anterior corner of eye; a short and narrow light yellow stripe present on tip of snout; a dark blotch present on upper lip under the eye; a distinct small dark spot present on between anterior corners of eyes; supratympanic fold and tympanic region slightly darker; a relatively large dark blotch present on above axilla; lower flanks scattered with irregular dark marbling. Ventral surface immaculate, violet-grey in color. Iris dark blackish, with light golden pigmentation relatively scarce on anterior, posterior, upper and lower corners, forming an indistinct X-shaped golden marking (Fig.3D).
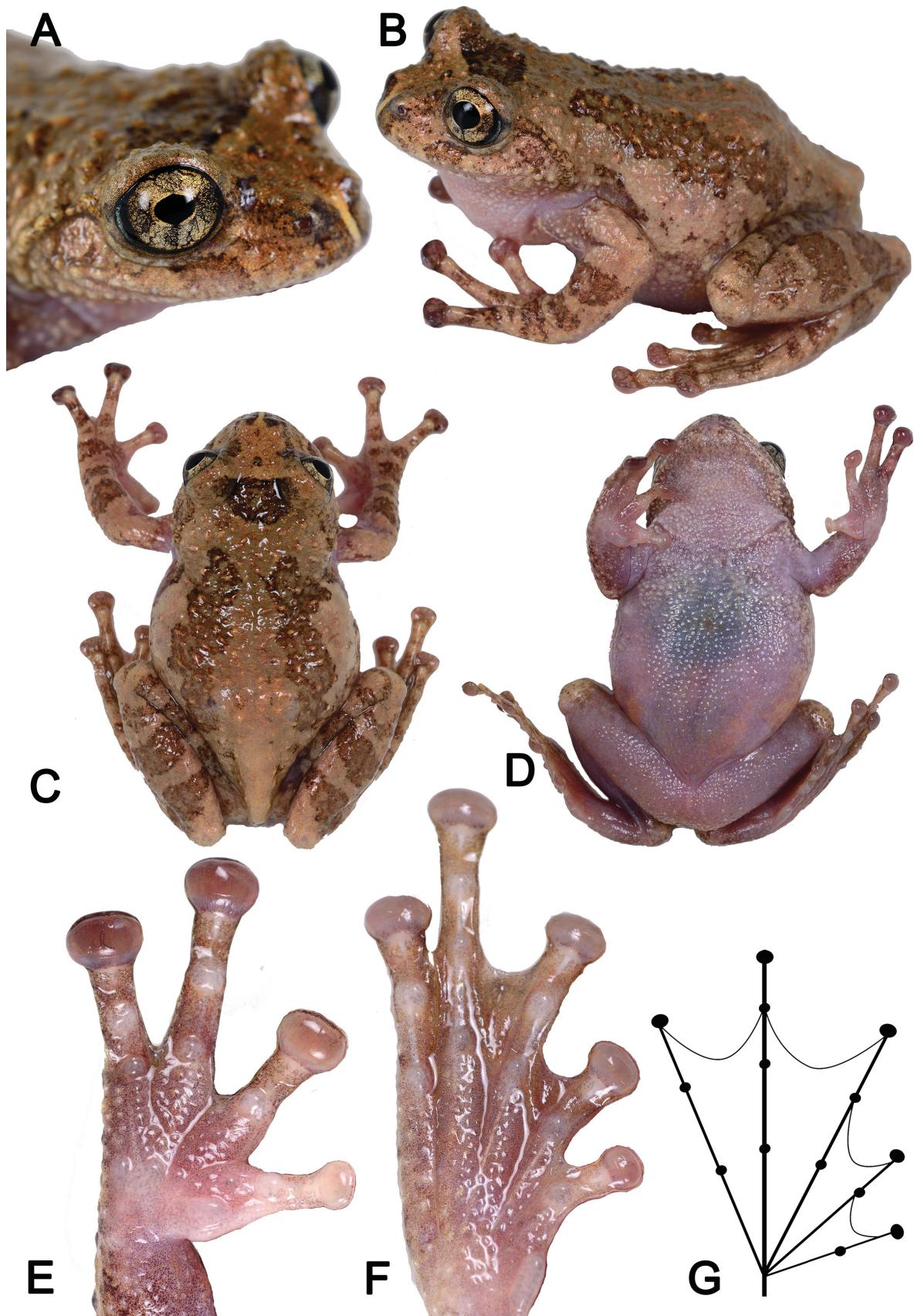
FIGURE 3. Holotype of *Nasutixalus yingjiangensis* **sp. nov.** (SYS a005802) in life: (A) anterolateral view of right head, noted the X-shaped iris pattern and raised canthus rostralis; (B) general view, dorsolateral aspect; (C) dorsal view; (D) ventral view; (E) thenar view of hand; (F) volar view of foot; (G) schematic illustration of foot webbing. Photos by J.H. Yang.
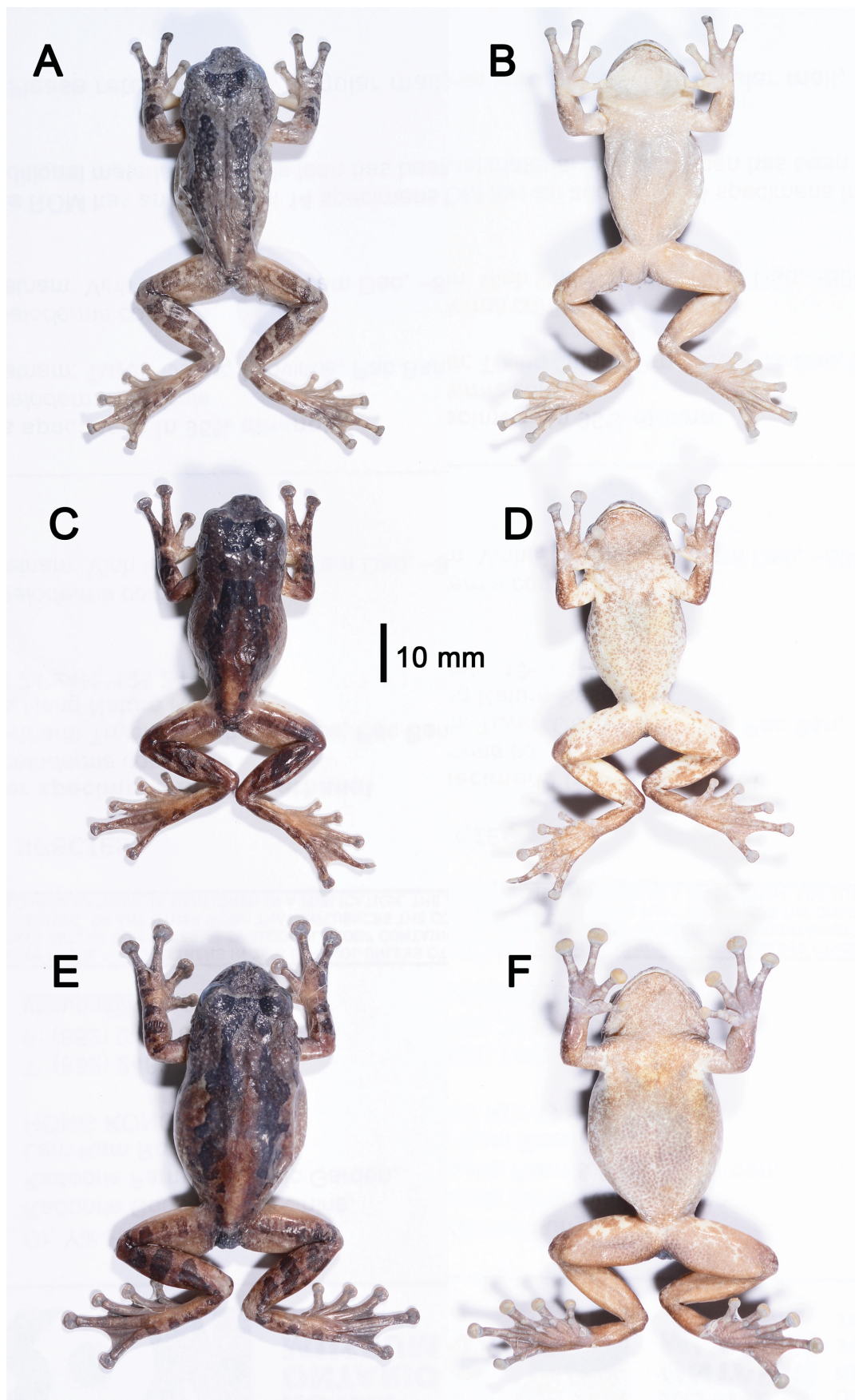
FIGURE 4. Types of *Nasutixalus yingjiangensis* **sp. nov.** in preservative: (A–B) holotype SYS a005802; (C–D) male paratype SYS a005803; (E–F) female paratype SYS a005804. Photos by J.H. Yang.

TABLE 2. Uncorrected *p*-distances (%) based on fragment of the 12S and 16S rRNA genes. (To be continued)

	(1–3)	4	5	6	7	8	9	10	11
(1–3) *Nasutixalus* sp.	0.0								
(4) *Beddomixalus bijui*	14.2	–							
(5) *Buergeria oxycephala*	18.5	16.9	–						
(6) *Chiromantis nongkhorensis*	15.9	17.1	21.5	–					
(7) *Feihyla palpebralis*	13.8	13.6	17.8	14.1	–				
(8) *Ghatixalus variabilis*	13.3	11.2	16.7	13.6	11.6	–			
(9) *Gracixalus gracilipes*	13.8	12.6	18.6	15.5	13.4	12.3	–		
(10) *Kurixalus odontotarsus*	14.1	15.2	21.9	17.0	16.5	15.0	15.3	–	
(11) *Liuixalus ocellatus*	13.1	14.6	16.9	15.1	11.7	13.1	12.6	16.3	–
(12) *Mercurana myristicapalustris*	16.7	15.5	20.1	18.3	17.0	14.3	15.8	11.0	16.6
(13–20) *Nasutixalus jerdonii*	7.1–7.5	15.3–15.8	19.5–20.2	16.3–16.7	15.4–15.6	15.7–16.2	14.4–15.1	16.5–17.0	14.6–14.9
(21–24) *Nasutixalus medogensis*	7.9	14.0–14.2	18.3–19.1	14.3–14.8	14.1–14.5	13.3–13.6	15.2–15.8	14.0–14.2	13.8–14.4
(25) *Nyctixalus pictus*	17.4	16.2	18.8	17.2	15.5	13.8	13.1	18.3	14.7
(26) *Philautus aurifasciatus*	13.1	13.0	19.0	15.9	13.5	14.0	14.6	17.8	13.8
(27) *Polypedates megacephalus*	17.3	19.9	22.7	15.9	17.0	16.0	18.1	19.2	16.2
(28) *Pseudophilautus microtympanum*	17.7	13.2	21.5	19.3	15.8	14.0	15.3	16.9	17.2
(29) *Raorchestes glandulosus*	17.7	15.4	19.9	17.2	14.9	14.7	15.6	18.8	17.5
(30) *Rhacophorus reinwardtii*	14.2	14.7	19.3	14.7	10.4	10.9	11.5	15.6	13.0
(31) *Taruga fastigo*	15.4	18.7	22.3	16.5	14.9	13.6	15.0	18.4	15.0
(32) *Theloderma asperum*	18.4	17.1	19.1	19.6	17.6	15.6	16.5	18.1	17.7
(33) *Limnonectes poilani*	22.6	21.6	22.4	23.0	19.6	21.4	20.6	25.7	19.2

TABLE 2. (Continued)

	12	(13–20)	(21–24)	25	26	27	28	29	30	31	32	33
(1–3) Nasutixalus sp.												
(4) Beddomixalus bijui												
(5) Buergeria oxycephala												
(6) Chiromantis nongkhorensis												
(7) Feihyla palpebralis												
(8) Ghatixalus variabilis												
(9) Gracixalus gracilipes												
(10) Kurixalus odontotarsus												
(11) Liuixalus ocellatus												
(12) Mercurana myristicapalustris	–											
(13–20) Nasutixalus jerdonii	18.8–19.3	0.0–0.8										
(21–24) Nasutixalus medogensis	15.0–15.7	9.8–10.2	0.0–1.6									
(25) Nyctixalus pictus	17.5	18.6–19.2	17.3–17.7	–								
(26) Philautus aurifasciatus	17.4	15.2–15.6	14.1–15.0	16.1	–							
(27) Polypedates megacephalus	20.5	18.9–19.6	16.5–17.2	19.1	17.6	–						
(28) Pseudophilautus microtympanum	14.3	18.3–18.6	16.9	19.4	15.3	20.8	–					
(29) Raorchestes glandulosus	16.5	19.0–19.6	17.2–18.2	18.0	16.4	18.0	13.9	–				
(30) Rhacophorus reinwardtii	14.7	16.0–16.7	13.7–14.0	14.6	14.0	17.1	14.7	14.3	–			
(31) Taruga fastigo	20.0	17.5–17.7	16.3–17.1	19.6	17.1	15.0	19.6	18.5	13.7	–		
(32) Theloderma asperum	17.3	19.1–19.5	17.5–17.9	15.6	17.7	21.7	18.2	19.4	17.5	19.6	–	
(33) Limnonectes poilani	25.7	23.5–23.9	23.9–24.3	22.5	21.7	24.3	22.1	23.5	21.1	22.7	23.3	–

Coloration of holotype in preservative. Greyish brown above, dark patterns on dorsum and bandings on limbs still visible; venter uniform grey, without distinct dark spots or marking; throat greyish white (Fig. 4). Light golden pigmentation of iris coloration fades to greyish white.

Variation. The male paratype SYS a005803 and female paratype SYS a005804 match the overall characters of the holotype (for measurements of the type series see Table 3). The two broad dark stripes in contact with each other anteriorly and separated from the dark marking on head in male paratype SYS a005803, while the female paratype SYS a005804 has the two broad dark stripes extend anteriorly and connect with the dark marking on head (see Fig. 5). A narrow, light yellowish stripe borders the anterior edge of the dark interorbital blotch in the female paratype SYS a005804 (Fig. 5C). In preservative, the ventral surfaces of body, thigh and tibia of male paratype SYS a005803 are dull white and scattered with distinct, small dark brown spots and marbling, while the female paratype SYS a005804 has an immaculate, dark brown venter.

Comparisons. For the two known members of the genus *Nasutixalus*, *N. yingjiangensis* **sp. nov.** differs from *N. medogensis* by having a relatively smaller body size (males SVL 39.5–40.0 mm vs. 45.0 mm), interorbital distance shorter than the upper eyelid width (mean male IOD/UEW ratio 87.9% in the new species vs. 110.3% in *N. medogensis*), a relatively shorter foot (mean male TFL/SVL ratio 67.5% in *Nasutixalus yingjiangensis* **sp. nov.** vs. 72.7% in *N. medogensis*), dorsal surfaces of head and the anterior part of body with dense and prominent tubercles (vs. relatively smooth in *N. medogensis*), and a different coloration pattern in life (dorsum of body and limbs without light green patterns in the new species vs. light green patterns present in *N. medogensis*). *Nasutixalus yingjiangensis* **sp. nov.** can be distinguished from *N. jerdonii* by having interorbital distance shorter than the upper eyelid width (mean male IOD/UEW ratio 87.9% in the new species vs. 121.2% in *N. jerdonii*), a relatively shorter foot (mean TFL/SVL ratio 67.0% and 62.9% in males and female respectively in the new species. vs. 70.6% (N=11) and 66.5% (N=1) in *N. jerdonii*), tympanum indistinct and covered with tubercles (vs. tympanum fully exposed and smooth in *N. jerdonii*), and nuptial pads only present on dorsal surface of finger I (vs. present on dorsal surface of finger I and finger II in male paratype of *N. jerdonii,* refers to Biju *et al.* 2016). Finally, the new species is distinct from all currently recognized species of *Nasutixalus* in the fragments of the 12S and 16S rRNA mt DNA genes examined (*p*> 7.1%).
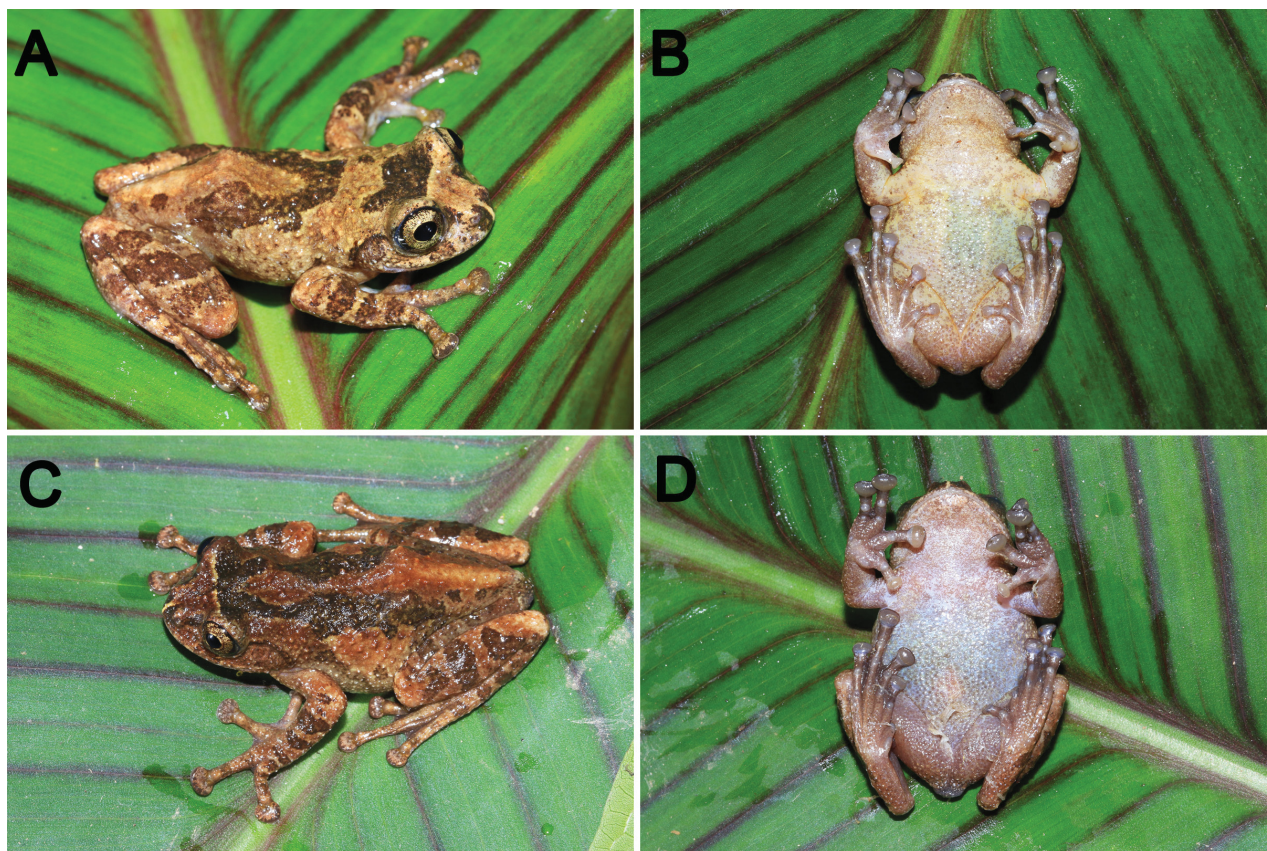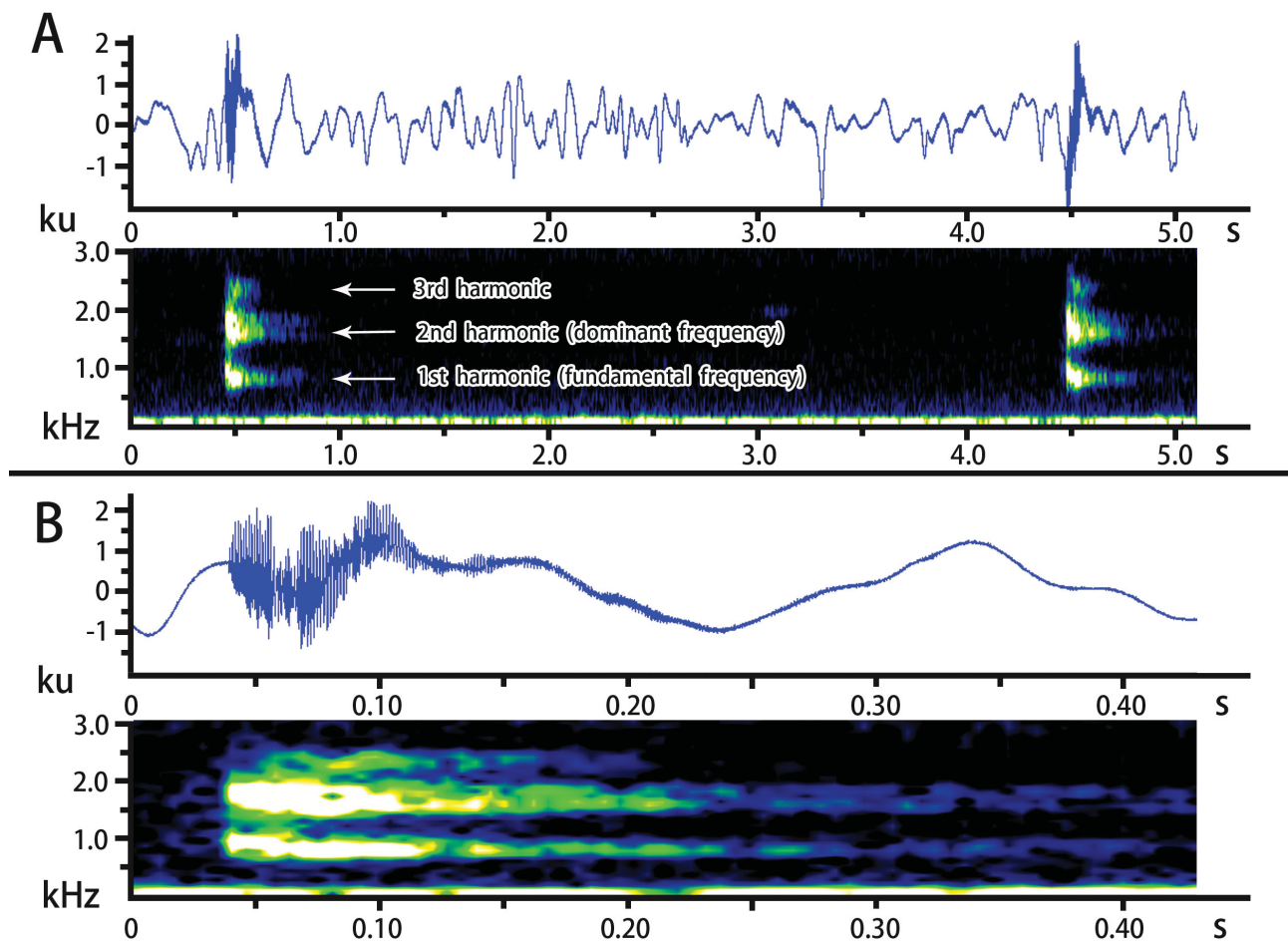

FIGURE 5. *Nasutixalus yingjiangensis* **sp. nov.**: (A–B) adult male paratype SYS a005803 in life; (C–D) adult female paratype SYS a005804 in life. Photos by J.H. Yang.
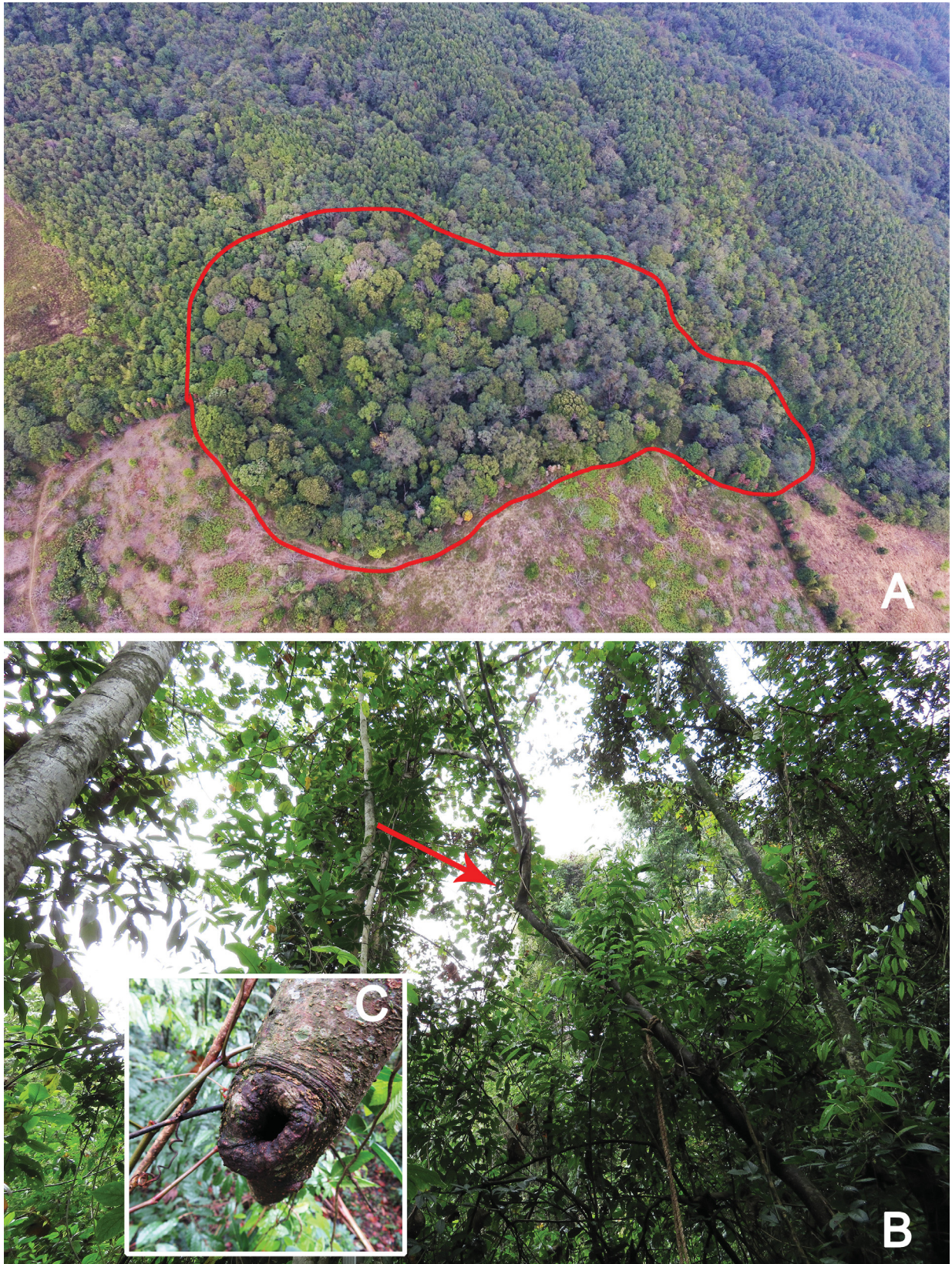
FIGURE 6. Habitat of *Nasutixalus yingjiangensis* **sp. nov.**: (A) type locality—mature evergreen montane forest fragment surrounded by farmland, cleared forest and *Betula alnoides* timber plantation; (B) microhabitat of the male holotype SYS a005802, red arrow denotes location of the tree hole, where the calling male holotype was found; (C) close-up of the tree hole opening located ca. 4.5 m above the forest floor. Photos by Joanne Li (A, B) and Zhi-Hua Zhang (C).

FIGURE 7. Advertisement call of an unvouchered male individual of *Nasutixalus yingjiangensis* **sp. nov.**: (A) waveform and spectrogram showing a two note portion of a call group; (B) waveform and spectrogram showing a single call.

Distribution and natural history. *Nasutixalus yingjiangensis* is currently only known from its type locality in Yingjiang County, western Yunnan, China. The type locality is a small, isolated patch of mature montane evergreen forest with an average canopy height of 30 m (Fig. 6); this forest remnant is surrounded by a mosaic of farmland, orchards, *Betula alnoides* timber plantation, and recently-cleared forest land.

The male holotype SYS a005803 was found calling inside a small tree hole on a *Tetradium glabrifolium* tree at 21:30 h on 20 April 2017 (Fig, 6B); it has rained during the day. The tree hole was about 4.5 m above the ground; the opening was small and oval in shape (ca. 18 mm in width and 25 mm in length), and the tree hole cavity had conical shape and was about 100 mm in depth, 30 mm at the widest and 15 mm at its narrow end. The single female paratype SYS a005804 was found and collected from inside a large tree hole which was about ca. 5 m above ground during the day survey on 11 June 2017; the opening was about ca. 80 mm in diameter, while the tree hole cavity was about ca. 120 mm in depth. The other male paratype SYS a005803 was also found and collected from inside a small tree hole which was about ca. 2 m above ground during the day survey; the opening was about ca. 40 mm in diameter, while the tree hole cavity was about ca. 80 mm in depth. These observations suggest that the new species is a phytotelm-breeder, similar to *N. jerdonii* (Biju *et al.* 2016).

We conducted four field surveys at the type locality between July 2016 and June 2017. Many males were heard calling from the canopy (mostly ca. 4 m above ground) during summer surveys on 5 May 2016, 20 April and 10 June 2017; no male calls were detected during the winter survey on 8 December 2016. This indicates the new species breeds during the rainy season starting from mid-April. Calling activity was highest from dusk until midnight. Sympatric rhacophorid treefrogs recorded in the forest fragment during the surveys include *Rhacophorus maximus* Günther, *Rhacophorus rhodopus* Liu & Hu, *Raorchestes longchuanensis* (Yang & Li), *Kurixalus odontotarsus* (Ye & Fei) and *Polypedates* cf. *braueri* (Vogt). No eggs and tadpoles of *Nasutixalus yingjiangensis* were found during the survey.

Advertisement call. The call series contains a single, pulsed call of 0.297–0.391 s duration (mean 0.344 ± 0.035 s, N=12). The intercall-interval is of 3.527–6.150 s (mean 4.195 ± 0.770 s, N=11). Calls are repeated in series at a rate of 13.3 times per minute on average. All calls contain a fundamental frequency and a dominant frequency. The dominant frequency range is of 1.56–1.64 kHz, whereas the fundamental frequency is of 0.76 kHz. A third harmonic is weakly present at 2.32–2.41 kHz (Fig. 7). To the human ear, the advertisement call of the new species is a short and low-pitched "uh".

TABLE 3. Measurements of types of *Nasutixalus yingjiangensis* **sp. nov.**, and comparison with two congeners *Nasutixalus jerdonii* and *Nasutixalus medogensis* (all measurements in mm).

	Nasutixalus yingjiangensis **sp. nov.**				*N. jerdonii*		*N. medogensis*
	SYS a005802	SYS a005803	mean ±SD (N=2)	SYS a005804	(N=1)	mean ±SD (N=11)	(N=1)
Sex	Male	Male		Female	Female	Male	Male
SVL	40.0	39.5	39.8±0.35	47.5	46.8	39.9±1.9	45.0
HL	13.8	14.1	14.0±0.21	16	15.7	13.2±0.6	15.5
HW	15.4	15.1	15.3±0.21	17.3	16.9	14.1±0.6	16.1
SL	5.8	5.7	5.8±0.07	6.6	6.7	5.6±0.2	6.8
IOD	3.7	3.5	3.6±0.14	3.7	4.7	4.1±0.1	4.3
IND	4.0	4.2	4.1±0.14	4.6	--	--	4.9
UEW	4.0	4.2	4.1±0.14	4.6	4.3	3.4±0.3	3.9
ED	5.4	5.8	5.6±0.28	6.2	5.3	4.8±0.4	6.0
TD	2.2	2.2	2.2±0.00	2.1	1.6	1.7±0.2	2.4
LAHL	20.6	20.8	20.7±0.14	23.6	--	--	24.4
FAL	--	--	--	--	9.8	7.9±0.6	--
HAL	13.1	13.3	13.2±0.14	14.7	15	13.1±0.7	14.9
FML	18.0	17.7	17.9±0.21	19.7	19.7	17.1±0.7	20.8
TIB	18.4	18.2	18.3±0.14	21.3	20.6	18.2±0.5	21.2
TFL	26.6	26.7	26.7±0.07	29.9	31.1	28.2±1.5	32.7
FL	18.9	19.1	19.00.14	21.2	22	19.0±0.9	22.1

Remarks: measurements of *N. jerdonii* and *N. medogensis* obtained from Biju *et al.* 2016 and Jiang *et al.* 2016 respectively.

Discussion

The discovery of *Nasutixalus yingjiangensis* greatly extends the distribution of the genus *Nasutixalus* from west Bengal, through northeast India and southeast Tibet, to western Yunnan of China (Fig. 1), and represents the third known species of this newly established genus. Due to their cryptic and arboreal habits, *Nasutixalus* species are difficult to detect and collect in the wild. Since the original description of *Polypedates jerdonii* Günther, 1876 (now *Nasutixalus jerdonii*) from "Darjeeling" in West Bengal, India, no additional specimens of this mysterious frog were reported for more than 130 years, until Indian herpetologists rediscovered the species from several locations in northeast India in 2007 and recognized it as *N. jerdonii* (Biju *et al.* 2016). It is noteworthy, that despite intensive field research efforts, further attempts to find this species in the environs of its type locality in Darjeeling have been unsuccessful (Biju *et al.* 2016). The two remaining members of the genus, *N. medogensis* and *N. yingjiangensis* were only discovered in the last two years (Jiang *et al.* 2016; this study).

The type locality of *N. yingjiangensis* is less than one kilometer from the boundary of Tongbiguan Provincial Nature Reserve, and ca. 5 km from the international border with Myanmar's Kachin State; we can expect that the new species also occurs in the well-preserved natural forests of Tongbiguan Nature Reserve and Kachin State of Myanmar. The extensive forests of northern Myanmar is biologically still insufficiently explored; it is likely that further survey efforts in northern Myanmar could lead to discovery of other *Nasutixalus* species in future.

During our survey, male calls of *N. yingjiangensis* were only detected in mature natural forest with complex, multi-layered vegetation, and no calls were heard in adjoining old-growth monoculture plantation forest, despite the fact that *Betula alnoides* is native to the region, suggesting that the new species is a forest-dependent species and only live in well-preserved natural forest. In Yingjiang County of Yunnan Province, the major threat to the local biodiversity is the clearance and degradation of natural forests for agricultural expansion, which severely fragments the natural forests and threatens forest-dependent species including *N. yingjiangensis*. The forest fragment of the type locality of *N. yingjiangensis* is now leased by a local conservationist who is determined to protect the site from further encroachment. The Hong Kong-based NGO Kadoorie Farm and Botanic Garden (KFBG), together with the Critical Ecosystem Partnership Fund (CEPF), are helping the landowner to preserve the site as a community-protected forest.

References

Biju, S.D., Senevirathne, G., Garg, S., Mahony, S., Kamei, R.G., Thomas, A., Shouche, Y.S., Raxworthy, C.J., Meegaskumbura, M. & Van Bocxlaer, I. (2016) *Frankixalus*, a new rhacophorid genus of tree hole breeding frogs with oophagous tadpoles. *PLoS ONE*, 11 (1), e0145727, 1–17.
https://doi.org/10.1371/journal.pone.0145727

Chen, X., Chen, Z., Jiang, J., Qiao, L., Lu, Y., Zhou, K., Zheng, G. & Liu, J. (2013) Molecular phylogeny and diversification of the genus *Odorrana* (Amphibia, Anura, Ranidae) inferred from two mitochondrial genes. *Molecular phylogenetics and evolution*, 69 (3), 1196–1202.
https://doi.org/10.1016/j.ympev.2013.07.023

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9 (8), 772.
https://doi.org/10.1038/nmeth.2109

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, 32, 1792–1797.

Fei, L., Hu, S.Q., Ye, C.Y. & Huang, Y.Z. (2009) *Fauna Sinica. Amphibia Vol. 2 Anura.* Science Press, Beijing, 957 pp. [in Chinese]

Frost, D.R. (2017) Amphibian Species of the World: an Online Reference. Version 6.0 Electronic Database accessible. American Museum of Natural History, New York, USA. Available from: http://research.amnh.org/herpetology/amphibia/index.html (accessed 9 May 2017)

Jiang, K., Yan, F., Wang, K., Zou, D. H., Li, C. & Che, J. (2016) A new genus and species of treefrog from Medog, southeastern Tibet, China (Anura, Rhacophoridae). *Zoological Research*, 37 (1), 15–20.

Rambaut A, Suchard M.A., Xie, D. & Drummond, A.J. (2014) Tracer v1.6, Available from: http://beast.bio.ed.ac.uk/Tracer (accessed 30 January 2018)

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61 (3), 539–542.

Savage, J.M. (1975) Systematics and distribution of the Mexican and Central American stream frogs related to *Eleutherodactylus rugulosus. Copeia*, 1975 (No. 2), 254–306.
https://doi.org/10.2307/1442883

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the entomological Society of America*, 87 (6), 651–701.
https://doi.org/10.1093/aesa/87.6.651

Sivongxay, N., Davankham, M., Phimmachak, S., Phoumixay, K. & Stuart, B.L. (2016) A new small-sized *Theloderma* (Anura: Rhacophoridae) from Laos. *Zootaxa*, 4147 (4), 433–442.

https://doi.org/10.11646/zootaxa.4147.4.5

Stamatakis, A. (2014) RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30 (9), 1312–1313.
https://doi.org/10.1093/bioinformatics/btu033

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30 (12), 2725–2729.
https://doi.org/10.1093/molbev/mst197

Vences, M., Thomas, M., van der Meijden, A., Chiari, Y. & Vieites, D.R. (2005) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, 2 (1), 5.